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Cooling to heat of fusion (HOF), followed by rapid rewarming, does not reduce the integrity of microvascular corrosion casts

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This study utilized microvascular corrosion casting techniques to evaluate changes in the microvascular patency of rat hindpaws cooled to four different subzero temperatures. Left hindpaws of anesthetized rats in group one were cooled to -5°C, in group two to -15°C, in group three to heat of fusion (HOF), and in group four to HOF and then to -15°C. Although freezing did not take place in the hindpaws of groups one and two, initiation of freezing in the tissues, as indicated by HOF, did occur in groups three and four. Cooled hindpaws were rapidly rewarmed. Right hindpaws served as controls. Microvascular corrosion casts were made from the left and right hindpaws of all animals. There was no significant difference when the mean cast weights of cooled hindpaws from groups one, two, and three were compared to the mean cast weights of their respective control hindpaws. In group four, there was a significant difference ($p<0.05$) when the mean cast weight of the cooled hindpaws

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(47.69mg (± SEM 9.05) was compared to that of the control hindpaws (80.63mg (± SEM 12.23). Since, in this acute experiment, a loss of vascular integrity occurred when the hindpaws in group four were cooled to -15°C after reaching HOF, the initiation of freezing, alone, was not sufficient to reduce mean cast weight.

Keywords: Frostbite; scanning electron microscopy; Capillaries; anatomy. (CTDC)

Cooling to heat of fusion (HOF), followed by rapid rewarming, does not reduce the integrity of microvascular corrosion casts

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Short Title: Heat of fusion does not reduce cast integrity

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This study utilized microvascular corrosion casting techniques to evaluate changes in the microvascular patency of rat hindpaws cooled to four different subzero temperatures. Left hindpaws of anesthetized rats in group one were cooled to -5°C, in group two to -15°C, in group three to heat of fusion (HOF), and in group four to HOF and then to -15°C. Although freezing did not take place in the hindpaws of groups one and two, initiation of freezing in the tissues, as indicated by HOF, did occur in groups three and four. Cooled hindpaws were rapidly rewarmed. Right hindpaws served as controls. Microvascular corrosion casts were made from the left and right hindpaws of all animals. There was no significant difference when the mean cast weights of cooled hindpaws from groups one, two, and three were compared to the mean cast weights of their respective control hindpaws. In group four, there was a significant difference ($p<0.05$) when the mean cast weight of the cooled hindpaws (47.69mg $\pm$ SEM 9.05) was compared to that of the control hindpaws (80.63mg $\pm$ SEM 12.23). Since, in this acute experiment, a loss of vascular integrity occurred when the hindpaws in group four were cooled to -15°C after reaching HOF, the initiation of freezing, alone, was not sufficient to reduce mean cast weight.

The degree of damage from frostbite is determined by a number of factors including the minimum temperature reached and the length of exposure (9, 11, 17). One of the benefits of the universally accepted treatment for frostbite, rapid rewarming administered immediately to the frozen extremity, is a reduction in the amount of time the tissue is subjected to subfreezing temperatures (9, 13). A theory on direct injury from slow freezing, as is usually found clinically, contends that, as ice crystals form in the extracellular space the increased osmotic pressure results in the withdrawal of water from the cells, thus making it available for the formation of additional ice crystals (1, 9, 16). Dehydration and toxic electrolyte concentrations then develop within the cell, creating the potential for irreversible destruction. Damage to blood vessels, which can ultimately result in the loss of distal nutritive flow, occurs primarily to the endothelial cells, and is characterized by vascular stasis and tissue swelling resulting from leakage of fluid and plasma proteins through the capillary walls (1, 2). This vascular injury is generally acknowledged to be the major factor in tissue loss from frostbite.

An evaluation of the vasculature from tissues cooled in a range of temperatures below zero should reveal the interval during which damage from freezing occurs. However, since the degree of damage from freeze injury appears to be related to the inception

and growth of ice crystals, supercooling without freezing should not effect vascular patency. The extent of vascular damage from a standard freeze injury has previously been demonstrated utilizing microvascular corrosion casting techniques in conjunction with scanning electron microscopy (7). In the current investigation, casting techniques were utilized to evaluate changes in the vascular networks of rat hindpaws cooled to four separate subzero temperatures and then rapidly rewarmed. This permitted the determination of the interval during which a quantifiable loss of vascular integrity occurred.

Materials and Methods

Male Sprague Dawley CD strain rats (Charles River Laboratories, Wilmington, MA) were housed in wire bottom rat cages with food and water available ad libitum. Fifty seven rats, weighing 346-410g, were placed in four groups; group one contained 13 animals, group two contained 12 animals, group three contained 20 animals and group four contained 12 animals. Prior to cooling, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (70mg/kg body weight); if necessary, an additional dose of one-third the initial dose was administered.

In all animals, temperatures were measured with 23-gauge needle thermocouples inserted 1 cm below the knee under the left and right gastrocnemius muscles and 29-gauge needle thermocouples inserted subcutaneously into the plantar surface of both hindpaws. Core temperatures were monitored with a rectal probe. Throughout the experiment, animals were kept warm by circulating 37°C water through tubing coiled around them. Immediately before the initiation of cooling, animals in all groups received an intravenous injection of 1ml sterile saline/kg through cannulas placed in the tail veins; this was done in order to permit a comparison between group four from this study and a corresponding group from an earlier study. The left hindlimbs were then sheathed and immersed in an alcohol bath to the distal end of the femur and cooled at an average rate of 1.0°C per minute (Fig. 1) by an external gravity fed solution of alcohol and dry ice. The hindpaws in group one were cooled to -5°C and those in group two were cooled to -15°C. The hindpaws in group three were cooled until freezing occurred, as indicated by the release of latent heat of fusion (HOF) generated by the formation of ice crystals. For those in group four, cooling continued after HOF until the temperature in the hindpaws fell to -15°C. When the cooling procedure was complete, the left hindlimbs were withdrawn from the cooling bath and rewarmed in a 40°C water bath until the temperature under the

gastrocnemius muscles reached 37°C. The right hindlimbs served as uninjured controls and were neither cooled nor rewarmed.

Following rewarming, all cooled hindpaws were withdrawn from the 40°C bath and the sheaths were removed. A midline incision, followed by dissection, exposed the abdominal aortas which were cannulated below the left renal blood vessels. A Harvard infusion pump was then used to flush the lower limbs with 1.0% heparin in saline; this was followed by fixation with 0.5% glutaraldehyde in cacodylate-sucrose. Preparation of microvascular corrosion casts and scanning electron microscopic examination proceeded as previously reported by Daum et al. (7).

All cast weights were calculated as mean ± standard error of the mean (± SEM). A two-sample t test was used to test for the difference between the two mean weights from the initial twelve animals and the subsequent eight animals in group three. The cast weights of the control and the cooled hindpaws from each group were analyzed for statistical significance using a paired-sample t test.

Results

The initiation of freezing, heat of fusion, in the tissues did not occur in group one, when hindpaws were cooled to -5°C, or in group two which was composed of animals whose hindpaws supercooled directly to -15°C. Hindpaws in both of these groups were rewarmed immediately upon reaching their respective temperatures. Hindpaws in group three were cooled until HOF developed; this occurred at an average temperature of -14.4°C. Although HOF, which is indicative of the initiation of ice crystal formation in the tissues, did develop in the cooled hindpaws from group three, which were rewarmed immediately, this group also exhibited no reduction in microvascular patency (Fig. 2). Scanning electron microscopic examination of casts prepared from cooled hindpaws of group three demonstrated an intricate microvascular network representative of normal vasculature (Fig. 3).

In groups one and two, there was no significant difference when the mean cast weights of the cooled hindpaws were compared to the mean cast weights of their respective control hindpaws. However, the mean weight of casts from the control hindpaws from the twelve animals in group three (53.66mg $\pm$ SEM 14.46) was significantly lower than that of the cooled hindpaws in the group (93.09mg $\pm$ 11.66). Since it was not clear why this reduction

occurred, and since the mean cast weight of the control hindpaws was lower than those of the control hindpaws from groups one (83.96mg $\pm$ 7.20), two (88.46mg $\pm$ 18.10), and four (80.63mg $\pm$ 12.23), an additional eight animals were used in group three to insure accuracy. The mean weight of the casts from the control hindpaws from these eight animals was 88.46mg $\pm$ 15.49. This mean weight was higher than the mean cast weight of the control hindpaws from the first twelve animals; however, the two mean cast weights were not significantly different. Although some variability is inherent in the process, technical problems in the initial group of twelve animals cannot be ruled out. The twelve animals that were initially cooled to HOF were combined with the subsequent group of eight animals to comprise one group of twenty animals (group three). The mean weight of casts from the control hindpaws in group three (67.58mg $\pm$ 11.10) remained lower than that of the cooled hindpaws in the group (89.22mg $\pm$ 8.19), although the difference was not statistically significant. When hindpaws in group four were cooled to HOF, which occurred at an average temperature of -14.6°C, and then cooled to -15°C, their mean cast weight (47.69mg $\pm$ 9.05) was significantly different ($p<0.05$) from the mean cast weight of the control hindpaws in the group (80.63mg $\pm$ 12.23) (Fig. 4). A substantial loss of vascular integrity was illustrated in the casts from the hindpaws of group four (Figs. 5 and 6).

Discussion

There was no loss of vascular integrity when hindpaws from group one were cooled to -5°C or when those from group two were supercooled to -15°C and rewarmed without reaching HOF. However, this was not unexpected, since it has been reported that supercooling phenomena can occur down to approximately -15°C without intracellular ice crystal formation, and possibly without tissue damage (9). Furthermore, in earlier studies, Bowers et al. had detected no morphological changes in the soleus muscles of mice whose limbs were supercooled to -13°C without freezing (3). In the current study, there was also no loss of vascular integrity when the hindpaws from group three were cooled to HOF and immediately rewarmed. Although ice crystal formation was initiated in the hindpaws from this group, rapid rewarming undoubtedly halted the further development of ice crystals and eliminated any formed at HOF. There was, however, a significant loss of vascular integrity when the hindpaws from group four were cooled to HOF and then to -15°C. Microvascular corrosion casts prepared from the cooled hindpaws of this group demonstrated the absence of continuity that is characteristic of freezing in this model (6, 7). If cold-induced vasospasm, which has been implicated in the development and sequelae of cold injuries (8), did develop, it was relieved in the

cooled hindpaws of groups one, two and three, although, it may have been responsible for the decrease in vascular patency observed in the casts of those from group four.

It is generally accepted that the primary vascular injury from frostbite is the result of endothelial cell damage (2, 3, 15). Casts from some of the hindpaws in group four display depressions which may reflect membrane alterations in the endothelial cells on the luminal surface of the vessels (Fig. 6). These juxtaposed indentations can be easily differentiated from the individual imprints of endothelial cell nuclei which have been observed on the surface of casts from both veins and arteries of normal vessels (4, 5, 12, 14). Arterial nuclear imprints are characterized by their oval, elongated appearance along the vessel axis and venous nuclear imprints are round (4, 5), while the depressions observed in this report display a distinct pattern that is not similar to either of the above.

It should be noted that the mean cast weights of the control hindpaws (80.63mg $\pm$ 12.23) and the cooled hindpaws (47.69mg $\pm$ 9.05) from group four correspond to those of an analogous group from a previous study by Daum et al. in which the mean cast weight for the control group was 80.80mg $\pm$ 10.18 and that of the cooled group was 45.25mg $\pm$ 10.75 (6). The animals in the current study received

intravenous saline injections merely to ensure a similarity of conditions to those from the earlier study.

In this study, cooling to HOF was not sufficient to reduce mean cast weight. Although the average total cooling times for group two to reach -15°C and group three to reach HOF were similar, 47.3 and 47.7 minutes respectively, the average total cooling time for group four was approximately 5 minutes longer (Fig. 1). Since a significant loss of vascular integrity in the cooled hindpaws of group four developed after the initiation of HOF, it appears that the freeze injury occurred in the 4 to 5 minute interval between that event and when the hindpaws were cooled to -15°C after reaching HOF. Ice crystal formation certainly continued, throughout this period, subsequent to the initiation of freezing and prior to rewarming. In their studies with rabbit hindpaws, Gildenberg and Hardenbergh did not consider freezing to be complete until the temperature curve showed a rapid drop after leveling off at approximately -0.5°C (10). In this acute study, consummation of freezing made the difference between loss of vascular patency in group four and retention of patency in group three. The precise point at which the damage occurred during the interval between HOF and HOF followed by cooling to -15°C could be determined, in fut e studies, by transmission electron microscopy. If the progression of damage relates to the development and growth of ice crystals,

this information would be useful in developing approaches which involve the interruption or delay of the freezing process.

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The investigators adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences, National Research Council. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Figure Legends

Figure 1

The average temperature is shown for the left hindpaws of groups 1 through 4. The rate of cooling was approximately 1.0°C/min. The characteristic change associated with heat of fusion is shown in groups 3 and 4.

Figure 2

Photograph of a microvascular corrosion cast from a cooled hindpaw from group three (HOF).

Figure 3

Scanning electron micrograph of a section of a microvascular corrosion cast from a cooled hindpaw from group three. A nuclear imprint from an endothelial cell on the luminal surface of the vein is shown (arrow). Bar = 20um.

Figure 4

Histogram of the weights in milligrams of the microvascular corrosion casts from the control and cooled hindpaws of groups one, two, three, and four. Mean $\pm$ SEM are given in each case. The starred means are significantly different from each other.

Figure 5

Photograph of a microvascular corrosion cast from a cooled hindpaw from group four (HOF and then cooled to -15°C).

Figure 6

Scanning electron micrograph of a section of a microvascular corrosion cast from a cooled hindpaw from group four. The outlines of depressed areas on the surface of the cast are shown (arrows). Bar = 20um.

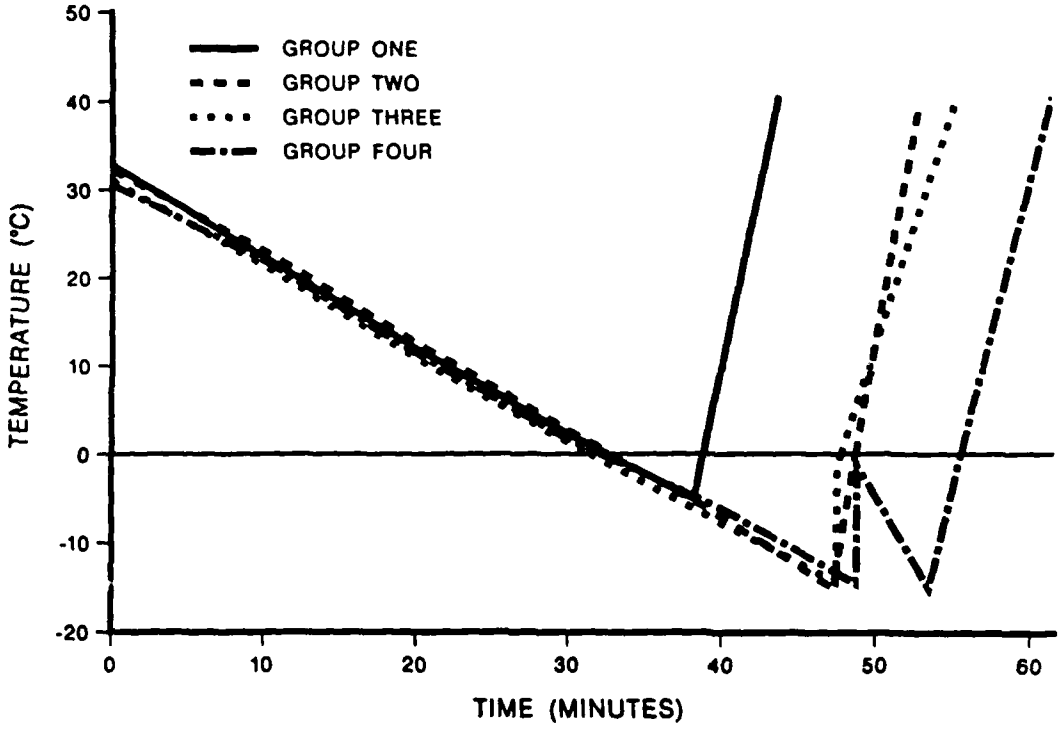
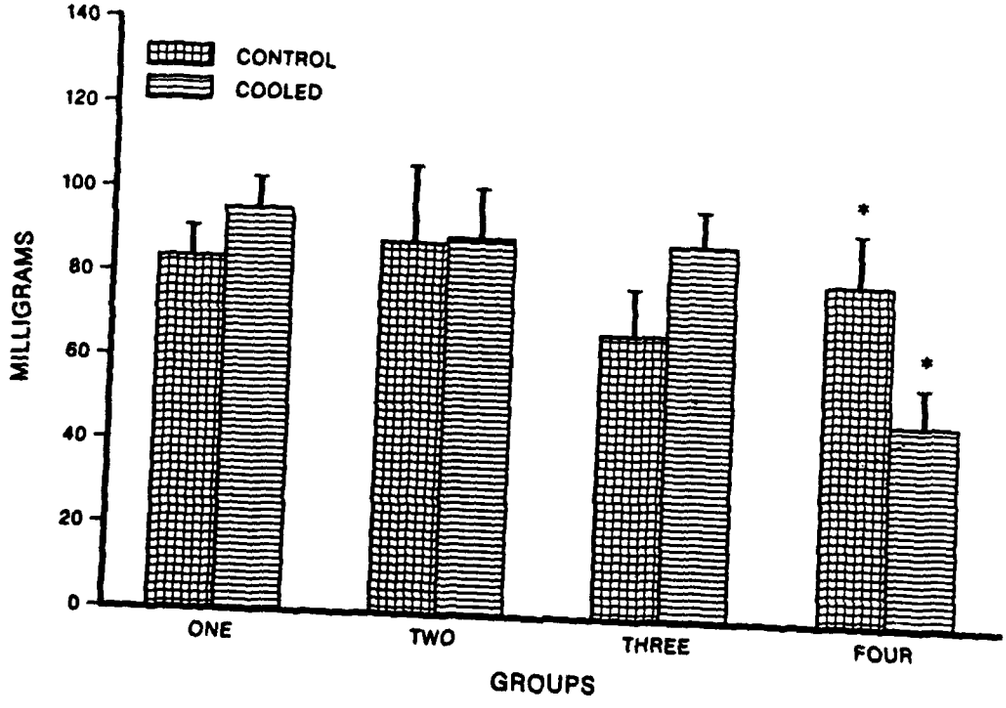

GROUP ONE
GROUP TWO
GROUP THREE
GROUP FOUR
TEMPERATURE (°C)
50
40
30
20
10
0
-10
-20
0
10
20
30
40
50
60
TIME (MINUTES)

Figure 1

Figure 2

Figure 3


140
120
100
80
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MILLIGRAMS
CONTROL
COOLED
*
*
ONE
TWO
THREE
FOUR
GROUPS

Fig 4

Fig 5

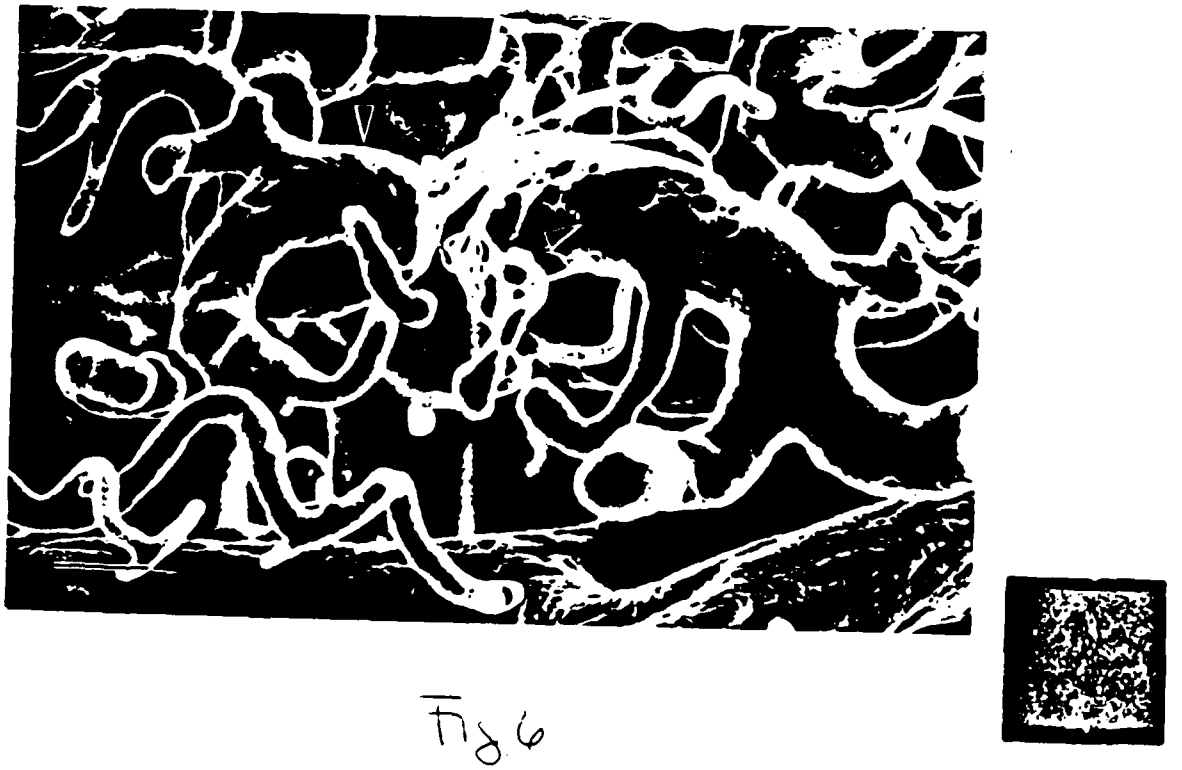

Fig 6